Immunity, Vol. 11, 225–230, August, 1999, Copyright ©1999 by Cell Press

Stat5 Activation Is Uniquely Associated with Cytokine Signaling in Peripheral T Cells

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Summary

The activation and subsequent proliferation of peripheral T cells requires the engagement of the T cell and a cytokine receptor, typically the IL-2 or IL-4 receptors. Critical to understanding the regulation of peripheral T cells is the knowledge of the unique contributions of each receptor to full T cell activation and cell cycle progression. Mice deficient in Stat5a and Stat5b have demonstrated the essential role that these highly related proteins play in cell cycle progression following peripheral T cell activation. Here we demonstrate that activation of the Stat5 proteins by tyrosine phosphorylation is uniquely contributed by cytokine receptor signaling and specifically does not occur through the T cell receptor complex.

Introduction

Activation of lymphocytes requires minimally two types of stimulation (Weiss and Littman, 1994). The first signaling events are initiated through recognition of antigen by the T cell receptor (TCR) complex. The consequences of TCR engagement are complex but require the activation of a series of protein tyrosine kinases including Zap70, Syk, Lck, and Fyn. One of the consequences is the induction of Ca^{2+} release, the activation of transcription factors, and the production of cytokines including IL-2 and IL-4. The second set of signals involve engagement of costimulatory receptors and the activation of cytokine receptors by their cognate cytokines. In peripheral T cells, IL-2 and, to a lesser extent, IL-4 play critical roles in T cell activation (Nelson and Willerford, 1998). Indeed, the absence of cytokine signaling during early T cell activation induces apoptotic T cell death or T cell anergy. Essential to understanding the regulation of peripheral T cells is the identification of the specific signals provided by the TCR and cytokine signaling complexes.

The derivation of mice lacking various genes has allowed the identification of critical signal-transducing proteins in T cell activation. In particular, we have recently derived mice that lack the genes for the two highly related signal transducers and activators of transcription (Stat) 5a and 5b (Teglund et al., 1998). The peripheral T cells from such mice are unable to proliferate in response to TCR engagement in the presence of IL-2 (Moriggl et al., 1999). Although Stat5-deficient peripheral T cells retain the ability to produce cytokines, the ability of IL-2 to induce the expression of cell cycle genes (*cyclin D2* and *D3* and *cdk6*) is lost, although p27 expression is downregulated normally. Other defects include the complete absence of NK cells, the presence of peripheral T cells with an activated phenotype, and the development of a pathology associated with extramedullary hematopoiesis. Collectively, the phenotype of Stat5a/b-deficient T cells is similar to that of T cells lacking the IL-2 receptor β chain (Suzuki et al., 1995, 1997).

Since the Stat5 proteins are essential for normal T cell activation, it becomes critical to determine whether their activation is mediated by kinases associated with the T cell receptor, cytokine receptors, or both. An early study examined the role of the IL-2 receptor and/or the TCR in the activation of the Jaks and Stats (Beadling et al., 1994). The results demonstrated that IL-2 receptor signaling, but not TCR signaling, activated Jak1 and Jak3. The only measure of Stat function examined in the study was the induction of DNA binding activity, since specific antibodies against most of the Stats were not yet available. Irrespective, the results demonstrated that IL-2 but not TCR signaling induced DNA binding activity against a Stat-binding site (SIE), but whether it was a known Stat protein could not be determined. More recently, it was reported that Stat5 proteins are transiently activated through TCR signaling in a T cell line, and this was attributed to the TCR-associated kinase Lck (Welte et al., 1999). Since potentially conflicting and incomplete results exist in the literature, we have examined in detail the question of whether cytokine signaling and/or TCR signaling activates Stat5 in normal peripheral T cells.

Results

We have previously shown that peripheral T cells lacking both Stat5 proteins but not the individual Stat5 proteins fail to proliferate in response to stimulation with anti-CD3 in the presence of IL-2. To determine whether the requirement for Stat5 activation applied to other cytokines, we examined the response to IL-4. IL-4 strongly and uniquely activates Stat6 (Hou et al., 1994), and the derivation of Stat6-deficient mice has demonstrated that Stat6 is essential for several IL-4 functions in late T cell differentiation including influencing Th2 differentiation and promoting immunoglobulin class switching to the production of IgE (Kaplan et al., 1996; Shimoda et al., 1996). However, IL-4 also induces the activation of Stat5a/b (Lischke et al., 1998) and it can be hypothesized that this function is important in peripheral T cell activation. This is supported by the results illustrated in Figure 1B. Peripheral T cells from wild-type, Stat5a-deficient, or Stat5b-deficient mice proliferated in response to limiting

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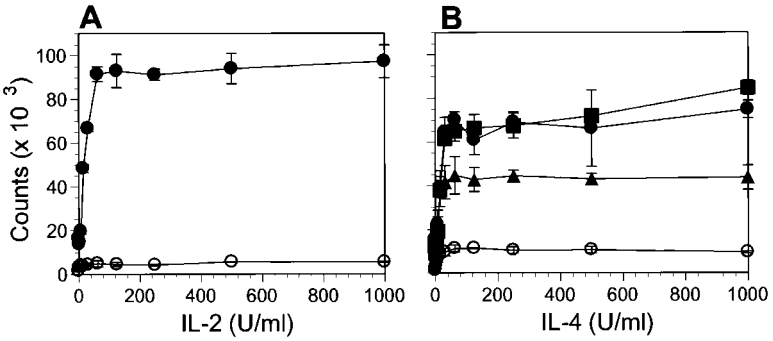

Figure 1. IL-4-Induced T Cell Proliferation Is Dependent upon Stat5 Proteins

Splenic lymphocytes from Stat5a (closed squares), Stat5b (closed triangles), or Stat5a/b deficient mice (open circles), as well as wild-type mice (closed circles), were cultured at a density of 1×10^5 cells per well in round-bottom 96-well plates for 72 hr in the presence of anti-CD3 with increasing concentrations, as indicated, of IL-4 (A) or IL-2 (B). The concentration of anti-CD3 utilized was sub-optimal for proliferation at a concentration of 0.5 μg/ml. The plates were assayed for proliferation by [^{3}H]thymidine incorporation for the last 18 hr of culture. The error bars reflect the results from six individual measurements.

concentrations of anti-CD3ϵ in the presence of IL-4. However, no proliferation was observed in Stat5a/b-deficient mice. For comparison, previous results comparing wild-type mice and Stat5a/b-deficient mice, obtained with anti-CD3 and increasing concentrations of IL-2, are also shown (Figure 1A). Therefore, the ability of IL-4 to activate Stat5a/b but not Stat6 is critical in the proliferative response of peripheral T cells to IL-4.

To investigate the roles of TCR and cytokine receptor function in Stat5 activation, peripheral T cells were isolated, expanded with anti-CD3ϵ and IL-2, and starved overnight. The cells were subsequently stimulated with anti-CD3ϵ, IL-2, or a combination of the two, and induction of tyrosine phosphorylation of TCR-associated chains determined. As illustrated (Figure 2A), stimulation of T cells with anti-CD3ϵ resulted in the rapid tyrosine phosphorylation of several TCR-associated chains including the ϵ and ζ chains. Of particular note is the appearance of p23, the hyperphosphorylated form of the ζ chain (Kersh et al., 1998). In contrast, IL-2 alone did not induce detectable, increased tyrosine phosphorylation of TCR-associated chains. Treatment of peripheral T cells with anti-CD3ϵ and IL-2 resulted in tyrosine phosphorylation of TCR chains that was comparable to anti-CD3ϵ alone. These results are consistent with a large literature demonstrating the unique ability of engagement of the TCR to induce tyrosine phosphorylation of the TCR-associated receptor chains (Alberola-Ila et al., 1997).

The patterns of Stat5 tyrosine phosphorylation, with the same lymphocyte extracts, are also shown in Figure 2. In contrast to TCR tyrosine phosphorylation, anti-CD3ϵ treatment did not result in the detectable tyrosine phosphorylation of either Stat5a (Figure 2B) or Stat5b (Figure 2C) at any of the times examined. In contrast, stimulation of T cells with IL-2 alone induced a rapid, strong, and sustained tyrosine phosphorylation of Stat5a and Stat5b. A comparable level of tyrosine phosphorylation of Stat5a and Stat5b was seen following stimulation with a combination of anti-CD3ϵ and IL-2. Identical results were obtained when cells were stimulated with IL-4 alone or in combination with anti-CD3ϵ, although the level of activation is significantly less than that seen with IL-2 (data not shown). To insure that the lack of detection of phosphorylated Stat5a/b was not due to the antisera used, we utilized a phospho-Stat5a/b antisera that had been previously shown to detect phosphorylation of Stat5a/b in D10 cells activated through the TCR (Welte et al., 1999). As illustrated in Figure 2D, this antiserum also failed to detect Stat5a/b activation by engagement of the T cell receptor in peripheral T cells but readily detected Stat5a and Stat5b activation in the response to IL-2.

The above studies were done with T cells that had been activated and expanded in tissue culture. We also studied the response of unexpanded peripheral T cells isolated from mesenteric lymph nodes. The cells were stimulated with IL-2 or anti-CD3ϵ alone or together, extracts were prepared, and a phospho-Stat5-specific antiserum was used in direct Western blotting. As illustrated in Figure 3, IL-2 strongly induced Stat5 phosphorylation that was detectable at 15 and 30 min. In contrast, treatment of T cells with anti-CD3ϵ did not induce detectable phosphorylation of Stat5 at either 15 or 30 min. The combination resulted in a level of Stat5 phosphorylation comparable to that seen with IL-2 alone. It should be noted that in experiments with lymphocytes isolated directly from animals, there is a weak and variable level of tyrosine phosphorylation of Stat5a/b that is seen at 30 min with anti-CD3ϵ. We attribute this to the production of cytokines that induce Stat5a/b tyrosine phosphorylation in T cells and as well as the non-T cells that exist in these populations.

The analysis of Stat5a/b tyrosine phosphorylation may not be sufficiently sensitive to detect a transient low level of activation. Another approach to assess Stat5a/b activation is induction of Stat5a/b DNA binding activity. As illustrated (Figure 4), treatment of expanded peripheral T cells with IL-2 rapidly induced a DNA binding complex that persisted throughout the times examined. In contrast, no DNA binding complexes were detectable in peripheral T cells treated with anti-CD3ϵ. Treatment with a combination of anti-CD3ϵ and IL-2 resulted in the induction of DNA binding complexes at levels comparable to those seen in cells treated with IL-2 alone. These results confirm the previous studies (Beadling et al., 1994). Since the previous studies did not assess the contribution of Stat5 to the complexes seen, the ability of antisera against Stat5 to supershift the complexes was examined. The complexes were completely supershifted by antisera against Stat5 but not with antisera against the other Stat family members (data not shown).

The above assays examined biochemical activation of Stat5 but did not address the possibility that a level

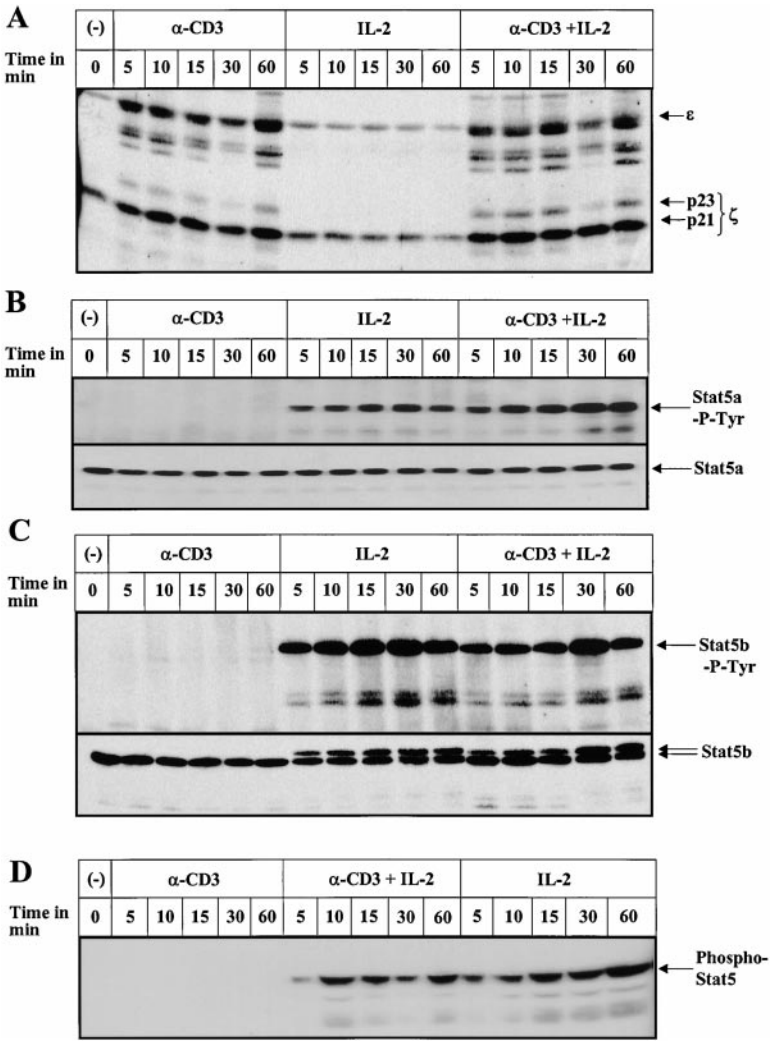

Figure 2. Tyrosine Phosphorylation of TCR Chains, Stat5a, and Stat5b in Activated T Cells

(A) Splenic T cells were stimulated with mAb 145.2C11 to CD3ϵ and IL-2 and expanded for 5 days. Cell purity was determined by flow cytometry to be >98% Thy1.2$^+$, ~60% CD8$^+$, and ~40% CD4$^+$. The cells were washed three times and starved for 24 hr (Moriggl et al., 1999) and either kept in media or stimulated with anti-CD3, anti-CD3, and IL-2, or IL-2 alone. At the indicated times, the cells were collected, added to excess ice-cold PBS, and lysed. The TCR complex was immunoprecipitated as previously described (Kersh et al., 1998) and as further described in Experimental Procedures. Tyrosine phosphorylation of the TCR ϵ and ζ chain (p21 and p23) was detected by the mAb 4G10 (Upstate Biotechnology).

(B and C) The same extracts were used for immunoprecipitation of Stat5a (B) or Stat5b (C) as described (Teglund et al., 1998) and the blots probed with an anti-phosphotyrosine monoclonal antibody (4G10, UBI). The membranes were stripped and reprobed (lower panels) with the mAb S21520 (Transduction Laboratories) specific to Stat5.

(D) At the indicated time points, T cells were lysed and whole-cell extracts were separated on 7.5% SDS-PAGE gels. Tyrosine phosphorylation of Stat5a and Stat5b was detected by the rabbit polyclonal antiserum PYS5 to phospho-Stat5ab (Zymed). Comparable results were obtained in three individual experiments.

of Stat5a/b activation by TCR engagement occurred that, while not sufficient for biochemical analysis, was sufficient for a biological response. One biological response of Stat5 activation is induction of Stat5 target genes including the cytokine-inducible SH2-containing gene *CIS* (Matsumoto et al., 1997). We therefore examined *CIS* induction following stimulation of the TCR and/or the IL-2 receptor. As shown (Figure 5A), *CIS* is strongly induced in peripheral T cells stimulated with IL-2. In contrast, *CIS* expression was not induced in peripheral T cells stimulated with anti-CD3ϵ alone. The combination of anti-CD3ϵ and IL-2 resulted in the induction of *CIS* expression at a level comparable to that observed in peripheral T cells stimulated with IL-2 alone.

Another approach of assessing the role of TCR signaling and IL-2 receptor signaling in Stat5a/b activation was to examine Jak3-deficient lymphocytes, since this kinase is uniquely activated by cytokine signaling (Nosaka et al., 1995; Thomis et al., 1995) and, as previously shown (Beadling et al., 1994), is specifically not activated by signaling through the TCR. Although young Jak3-deficient mice have reduced T cells, with age the numbers increase to levels comparable to wild type. As illustrated in Figure 5B, treatment of lymphocytes from wild-type mice with anti-CD3ϵ and IL-2 rapidly induced *CIS* expression as well as *IFN*γ expression. Consistent with a requirement for Stat5a/b activation, *CIS* induction was not observed in lymphocytes from Stat5a/b-deficient mice. Note that the weak band seen in this experiment at 4 hr has not been seen in other experiments. More importantly, no *CIS* induction was seen in lymphocytes from mice lacking Jak3. In contrast, the induction of *IFN*γ expression is observed in lymphocytes from both Jak3-deficient and Stat5a/b-deficient mice. Therefore, there are no signaling events from the TCR, even in the presence of IL-2 and its receptor, that can compensate for Jak3 in the functional activation of Stat5a/b. However, the TCR signaling events involved in cytokine production are unaffected by the absence of either Jak3 or Stat5a/b. Therefore, we conclude that Stat5 activation in peripheral T cells is uniquely associated with cytokine signaling.

Discussion

Our studies have utilized several biochemical and functional approaches to establish the specificity with which Stat5 is activated in peripheral T cells by the T cell receptor complex or by cytokine signaling. With all the

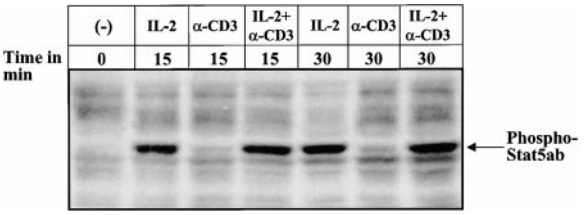

Figure 3. Stat5 Is Rapidly Tyrosine Phosphorylated in Normal, Unstimulated Peripheral T Cells

T cells from mesenteric lymph nodes of 10-week-old wild-type animals were isolated. FACS analysis of the cells indicated that approximately 50% were Thy1.2^{+}. The cells (1×10^6/ml) were stimulated with 1000 U/ml of IL-2 to bypass the requirement for the upregulation of the α chain of the receptor, anti-CD3 (2C11, 3 μg/ml), or a combination of the two stimuli. The proliferation was assessed as in Figure 1 and significant proliferation was only observed in the presence of both IL-2 and anti-CD3 (data not shown). After the indicated times, the cells were collected and lysed as described in the legend of Figure 2. Tyrosine phosphorylation of Stat5a and Stat5b was detected by a rabbit polyclonal antibody (PYS5) to phospho-Stat5. Note that the nonspecific bands indicate comparable loading of the samples. Comparable results were obtained in four individual experiments.

approaches, we conclude that the activation of Stat5a/b in peripheral T cells occurs uniquely through cytokine receptors and, specifically, does not occur through the T cell receptor complex. Since we have previously shown that the Stat5a and Stat5b proteins are redundantly critical for the proliferation of peripheral T cells in response to IL-2 or IL-4, as shown here, the results establish another essential function that cytokines uniquely provide to the regulation of peripheral T cells.

Our results differ from recently reported studies with the D10 T cell line (Welte et al., 1999). It should be noted, however, that the reported Stat5a/b activation in D10 cells was weaker than with IL-2 and transient. This is in contrast to normal peripheral T cells, as demonstrated here, in which IL-2 strongly activates Stat5 proteins over sustained periods of time. Much of the studies, however, have focused on biochemical evidence of Stat5a/b activation. It is clearly also important to seek functional evidence of Stat5a/b activation. As illustrated here, induction of the Stat5a/b-regulated gene *CIS* is readily detectable following IL-2 stimulation of peripheral T cells, while no induction is seen in cells stimulated through the TCR. Whether the level of Stat5a/b activation observed in D10 is sufficient to induce *CIS* expression has not been examined.

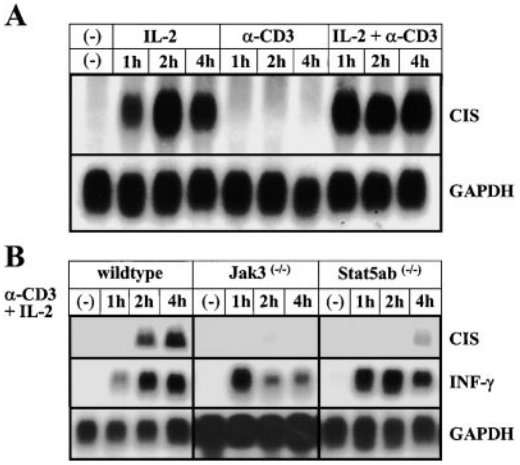

Figure 5. Expression of *CIS*, a Stat5a/b Target Gene

(A) Activated T cells were cultured, starved, and stimulated as in Figure 2 and RNA was isolated by RNazol (Tel-Test) after the indicated times. The RNA was separated on 1% agarose gels (20 μg/lane). A murine 0.2 kb BamHI–HindIII cDNA fragment for *CIS* and a 1.2 kb EcoRI fragment for *GAPDH* as loading control were labeled by [α-^{32}P]dCTP using a random labeling kit (Amersham). The membrane was hybridized using Rapid hybridization solution (Amersham) followed by stringent washes (final wash: 0.2 × SSC/0.1% SDS at 65°C). Similar results were obtained in three experiments.

(B) *CIS* induction in T cells from wild-type, Stat5ab-, and Jak3-deficient T cells. Comparable numbers of splenic T cells were stimulated with anti-CD3ε and IL-2. Total RNA was analyzed as in (A). All exposures were of comparable duration.

The level of TCR-induced transient Stat5a/b activation seen in D10 cells was more than we have observed in peripheral T cells. One possible explanation for the differences may be the fact that D10 cells are a long-term established T cell line, and, consequently, it is possible that other cell lines may exhibit similar properties. Another factor that may contribute to differences between D10 cells and normal T cells is their unique ability to produce copious amounts of IL-4 (Kupper et al., 1987) and, indeed, early lines relied on IL-4 as an autocrine growth factor. It has previously been demonstrated that IL-4 mediates activation of Stat5a/b (Lischke et al., 1998) and, as we have shown here, the ability of IL-4 to activate Stat5 proteins is critical for its contribution to the proliferation of peripheral T cells.

As our results illustrate, anti-CD3ε induced tyrosine phosphorylation of multiple chains of the T cell receptor in normal peripheral T cells, consistent with previous studies as reviewed in Alberola-Ila et al. (1997). The

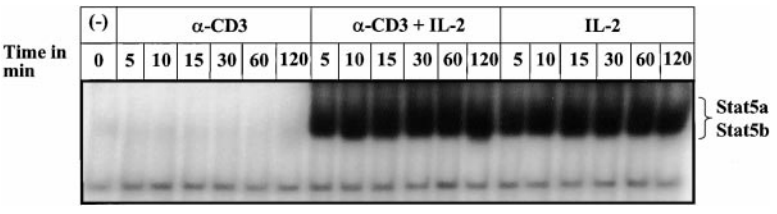

Figure 4. Stat5 DNA Binding Activity and Tyrosine Phosphorylation Are Not Coupled to TCR Activation

Activated T cells were either nonstimulated or activated with anti-CD3 and/or IL-2 for the indicated times. T cells were isolated, starved, stimulated, and harvested as described in Figure 2. (A) Whole-cell extracts from T cells were prepared and analysed by in vitro DNA binding assays using a double-stranded γ-^{32}P-labeled DNA probe comprising the Stat5 site from the β-casein promoter (Moriggl et al., 1996). Supershift experiments were carried out to verify specificity of the Stat5a/b-containing complexes (data not shown). Similar data were obtained in three independent experiments.

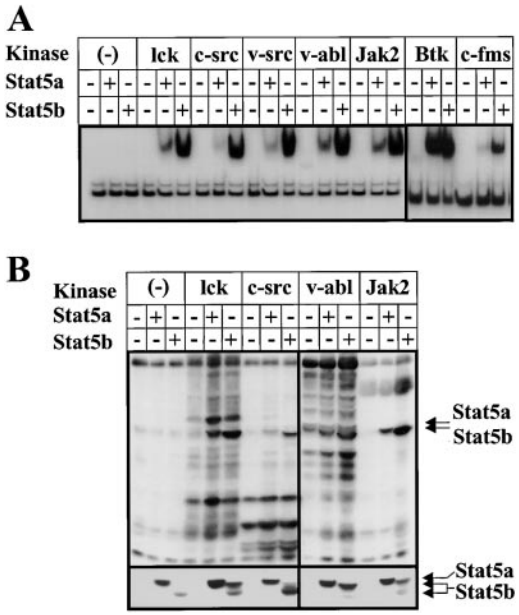

Figure 6. Overexpression of Several Tyrosine Kinases and of Stat5 Results in Stat5 Tyrosine Phosphorylation

293T cells were transfected by calcium phosphate precipitation with the indicated tyrosine kinases (4 μg/transfection) and/or Stat5a or Stat5b (4 μg/transfection) expression vectors (Moriggl et al., 1996). A β-galactosidase (1 μg/transfection) vector was included for normalization. 293T whole-cell extracts were analyzed for DNA binding (A) or for tyrosine phophosphorylation (B). Membranes were reprobed with the mAb S21520 specific to Stat5a/b (C).

phosphorylation of T cell receptor chains is mediated by one or more of several kinases associated with the TCR that, through derivation of deficient mice, have been shown to be critical for T cell differentiation. For example, the defects associated with a deficiency of Lck are early in T cell development (Molina et al., 1992). Importantly, this contrasts with a deficiency of IL-2 or Stat5a/b that only affecta the function of differentiated peripheral T cells.

Although not evident in our studies, previous studies have proposed a role for Lck in TCR-induced phosphorylation of Stat5a/b based largely on cotransfection approaches (Welte et al., 1999). Because we were unable to observe Stat5a/b phosphorylation under conditions in which Lck would be activated, we repeated the cotransfection studies. As illustrated (Figure 6), cotransfection of Lck with Stat5a or Stat5b in 293T cells resulted in tyrosine phosphorylation of Stat5a and Stat5b as well as the tyrosine phosphorylation of a number of proteins. Moreover, tyrosine phosphorylation was associated with the activation of DNA binding activity. Thus Lck can directly or indirectly induce Stat5 phosphorylation in cotransfection experiments. To assess the specificity, we examined additional tyrosine kinases. As illustrated in Figure 6, tyrosine phosphorylation and induction of DNA binding activity was observed in cells cotransfected with the Stat5 genes and all the kinases examined including c-Src, v-Src, v-Abl, Jak2, Btk, and c-Fms. These observations would suggest that overexpression in cotransfection experiments in 293T cells cannot be used to study the specificity of kinases for potential substrates nor can it be used as a model system for reconstituting T cell–specific signal transduction pathways.

Previous studies (Welte et al., 1999) detected Stat5a/b in immunoprecipitates of the T cell receptor complex in D10 cells and in T cells from a TCR transgenic strain of mice. Whether Stat5 proteins were tyrosine phosphorylated in response to antigen in the latter case was not examined. Based on these observations and cotransfection studies in 293T cells, it was proposed that the Stat5 proteins are specifically recruited to the T cell receptor complex by SH2 interaction with the ζ chain. Based on these observations, we have examined immunoprecipitates of the T cell receptor complex such as those shown in Figure 2A with various antisera against Stat5. In none of our experiments have we detected Stat5 proteins in the immunoprecipitates. The basis for the differences are not known.

In summary, our studies were designed to investigate the signaling pathways that are involved in the activation of the Stat5 proteins in peripheral T cells based on the critical role that they play in T cell proliferation. The studies demonstrate that activation of the Stat5 proteins is not mediated by signaling through the T cell receptor complex but rather is uniquely contributed by signaling through cytokine receptors such as IL-2 or IL-4.

Experimental Procedures

Splenic T cells or lymph node cells were isolated from 8- to 12-week-old wild-type mice with the same genetic background as the Stat5a/b- and Jak3-deficient mice as previously described (Moriggl et al., 1999). The properties of the Stat5a/b-deficient (Teglund et al., 1998) and Jak3-deficient (Nosaka et al., 1995) mice have been described in detail. Stat5a/b-deficient mice that had overt splenomegaly were excluded from any of the studies. Two- to three-month-old Jak3-deficient mice were used to obtain sufficient peripheral T cells to do the experiments.

For all the stimulation conditions, 5×10^6 T cells/ml were used for stimulation. IL-2 (Boehringer Mannheim) induction was carried out with 500 U/ml unless otherwise indicated, while IL-4 (RandD) was used at 100 ng/ml. For plate-bound anti-CD3ε (145.2C11), 10 μg/ml in PBS containing 0.2% BSA was used to coat tissue culture dishes for 18 hr at 4°C. The cross-linking of the TCR of naive T cells was carried out with anti-CD3ε (145.2C11) at 3 μg/ml in suspension culture media.

For the immunoprecipitation studies, harvested cells were washed twice and cell lysis was carried out on ice for 1 hr in a buffer containing 0.5% NP-40, 0.5% Tween-20, 25 mM Tris-HCL (pH 7.5), 25 mM HEPES buffer (pH 7.5), 75 mM NaCl, 10 mM β-glycerophosphate, 10 μM EDTA, 10 μg/ml leupeptin and aprotinin (both from Sigma), 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM PMSF. The further preparation of the samples, electrophoresis, and transfers were as previously described in detail (Moriggl et al., 1999).

Acknowledgments

The authors would like to thank Kristen Rothammer for excellent technical assistance. We would like to thank John Cleveland, Mary Ellen Conley, Haiyan Liu, Juerg Rohrer, Martine Roussel, and Dario Vignali for expression vectors for c-Src, v-Src, Btk, c-Fms, and Lck.

Received June 16, 1999; revised July 28, 1999.

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